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Alpha-Galactosyl trisaccharide epitope: Modification of the 6-primary positions and recognition by human anti-αGal antibody

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Galactose oxidase (EC 1.1.3.9, GAO) was used to convert the C-6′ OH of Galβ(1 → 4)Glcβ–OBn (5) to the corresponding hydrated aldehyde (7). Chemical modification, through dehydratative coupling and reductive amination, gave rise to a small library of Galβ(1 → 4)Glcβ–OBn analogues (9a–f, 10, 11). UDP-[6-^{3}H]Gal studies indicated that α1,3-galactosyltransferase recognized the C-6′ modified Galβ(1 → 4)Glcβ–OBn analogues (9a–f, 10, 11). Preparative scale reactions ensued, utilizing a single enzyme UDP-Gal conversion as well as a dual enzymatic system (GalE and α1,3GalT), taking full advantage of the more economical UDP-Glc, giving rise to compounds 6, 15–22. Galα(1 → 3)Galβ(1 → 4)Glcβ–OBn trisaccharide (6) was produced on a large scale (2 g) and subjected to the same chemoenzymatic modification as stated above to produce C-6″ modified derivatives (23–30). An ELISA bioassay was performed utilizing human anti-αGal antibodies to study the binding affinity of the derivatized epitopes (6, 15–30). Modifications made at the C-6′ position did not alter the IgG antibody's ability to recognize the unnatural epitopes. Modifications made at the C-6″ position resulted in significant or complete abrogation of recognition. The results indicate that the C-6′ OH of the αGal trisaccharide epitope is not mandatory for antibody recognition.
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Cell surface carbohydrates play essential roles in many normal and pathological biological recognition processes [1]. Considerable effort has been directed toward (i) understanding how carbohydrates function as recognition signals, and (ii) developing strategies to block undesirable interactions between cell surface carbohydrates and their protein targets. Ligands, that bind to their protein targets better than the natural cell surface carbohydrates could be effective in preventing or treating various diseases [2–6]. However, progress in understanding how structure and function are related in biologically active carbohydrates has been slow because obtaining synthetic carbohydrate derivatives for biochemical studies is difficult. In contrast, excellent chemical and biological methods to obtain large quantities of peptides and nucleic acids have been available for decades [7,8].

αGal epitopes [Galα(1 → 3)Gal-terminated oligosaccharide sequences including di- (**1**), tri- (**2** and **3**), and penta-saccharides (**4**)] have drawn increasing attention since the discovery that the interaction of preexisting natural antibodies in human serum with this oligosaccharide sequence on animal cells is the main cause of hyperacute rejection (HAR) in xenotransplantation (Figure 1) [9–11]. αGal epitopes exist as glycolipids or glycoproteins on the cell surface of mammals other than humans, apes, and Old World monkeys. The unique enzyme, responsible for the formation of the terminal glycosidic bond in nature, is α1,3-galactosyltransferase (α1,3GalT), a protein that is absent in humans due to mutational inactivation of the gene [12]. In contrast, humans produce a large amount of anti-αGal antibodies including IgG, IgM, and IgA isotypes. The discovery of the interaction of anti-αGal and αGal epitopes has led to attempts to overcome HAR rejection by either depleting the recipient's anti-αGal through αGal-immobilized affinity columns or antagonizing anti-αGal by infusing the recipient's body with soluble synthetic αGal oligosaccharides.

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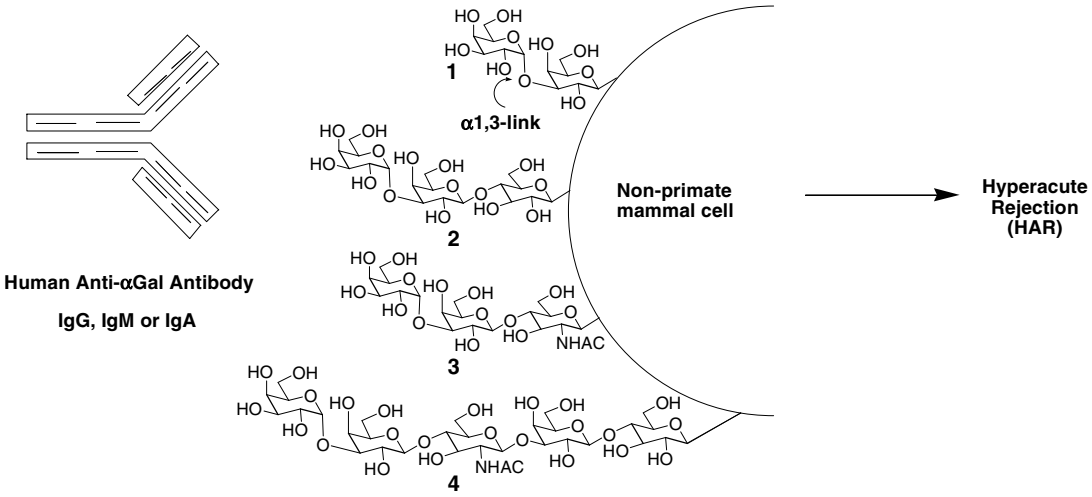


Figure 1. Human anti-αGal antibody recognizes Gal$\alpha(1 \rightarrow 3)$Gal linkage to invoke hyperacute rejection.

The recent interest in the preparation of natural and modified oligosaccharide structures stems from numerous demonstrations of their potential as pharmaceuticals [13]. One of the most important features of carbohydrate-protein binding is that the recognition process is generally initiated from the nonreducing end of the oligosaccharide and only part of the structure, usually one terminal sugar unit, is deep in the binding pocket in intimate contact with the protein. This specific sugar unit is called "immunodominant sugar" with regard to antibody binding. For αGal epitopes, the terminal Gal$\alpha(1 \rightarrow 3)$Gal is the key sugar unit that is recognized by anti-αGal antibodies and plays a crucial role in αGal/anti-αGal binding. The other sugar residues provide additional interactions with the antibody and enhance the overall binding affinity of αGal.

In the present work, we extend previous studies on the α1,3GalT-catalyzed synthesis of oligosaccharide analogues to include chemoenzymatic modified acceptors, which potentially can interfere with the binding site of the enzyme [14]. This enzyme transfers sugars with the net retention of configuration. There are two reasons why the use of enzymes in oligosaccharide synthesis has recently gained a high level of interest: (1) the enzymatic synthesis of a glycosidic linkage is always stereospecific and (2) no lengthy protecting and deprotecting schemes are required. The analogues were prepared by an enzymatic oxidation reaction, utilizing galactose oxidase (EC 1.1.3.9, GAO), which recognizes terminal galactose moieties and converts the primary hydroxyl group to the corresponding aldehyde in nearly quantitative yields. This aldehyde was then modified under aqueous conditions, exploring such transformations as dehydratative formation of oximes, reductive amination, and simple reduction to produce amines. The analogues were then tested for their binding affinity toward human anti-αGal antibodies. Thus, our design of αGal analogues allows for the terminal Gal$\alpha(1 \rightarrow 3)$Gal linkage to retain the binding specificity for both the transferase and the antibody, while derivatizing the C-6$'$ and C-6$''$ hydroxyl groups of the αGal trisaccharides.

Materials and methods

^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 MHz, VXR400 NMR and a Varian Unity 500 MHz spectrometer. Mass spectra (ESI, FAB, EI) were run on a Kratos MS-80 and a Kratos MS-50 instrument at Wayne State University. Thin-layer chromatography was conducted on precoated Whatman K6F silica gel 60 Å TLC plates with a fluorescent indicator. EM Science silica gel 60 Geduran (230–400 Mesh) was used for column chromatography. Optical absorption data for the ELISA assays were obtained on a BioRad microplate reader model 550 at wavelengths of 650 nm and 450 nm. Type AB human serum, peroxidase conjugated goat anti-human IgG antibody, and mouse laminin (a basement membrane glycoprotein containing 50–70 αGal epitopes per molecule) were purchased from Sigma. Size exclusion chromatography was performed on Biogel P2 and on Sephadex G-15 using distilled water as the eluent. Other reagents were obtained from commercial sources. Gal$\beta(1 \rightarrow 4)$Glcβ–OBn (**5**) was previously synthesized [15,16]. All other chemicals, including UDP-Glucose, UDP-Glactose, UDP-D-[6-^{3}H]Glucose, ampicillin, and DOWEX 1 $\times$ 8 anion-exchange resin were purchased from Sigma Chemical Co. (St. Louis, MO). Deionized water was obtained with a Water Pro PS system (Labconco, Kansas, MO).

Plasmids and strains

The construction of expression plasmid pET15b-α1,3GalT and pET15b-GalE, and the preparation of *E. coli* strain BL21(DE3) harboring the corresponding plasmids have been previously reported [14,17]. Briefly, the gene of interest was amplified by PCR and inserted into the NdeI and BamHI restriction sites of a pET15b plasmid vector. The resulting plasmids were then transformed into *E. coli* DH5α (cloning host) and BL21(DE3) (expression host).

Media and culture conditions

Recombinant *E. coli* strains harboring either plasmid pET15b-GalE or pET15b-α1,3GalT were cultivated in Luria-Bertani (LB) media containing 150 μg/mL ampicillin at 37°C in an incubator shaker (250 rpm) and induced with 0.4 mM IPTG (isopropyl-1-thio-D-galactopyranoside) for 3 h. The cells were isolated by centrifugation and stored at −20°C.

Preparation of α1,3GalT from transformed *E. coli* strain

Cells were cultured in LB medium containing 100 μg/mL ampicillin with rapid shaking (250 rpm) at 37°C in a C25 incubator shaker. The cultures were monitored by absorbance at 600 nm using a Beckman DU-600 spectrometer. When the *A*600 nm of the culture reached 0.8–1.0, IPTG was added to a concentration of 400 μM to induce the expression of α1,3GalT. After shaking at 37°C (250 rpm), the cells were harvested by centrifugation at 4000 rpm for 20 min and washed with washing buffer (pH 8.5, 20 mM Tris-HCl, 20% sucrose). Lysis buffer (pH 8.5, 20 mM Tris-HCl, 1 mM EDTA, 1% Triton × 100, 200 μg/mL lysozyme) was added, and the mixture was stirred vigorously for 10 min at room temperature. DNaseI (2 μg/mL) was added. The mixture was shaken at 37°C in a water bath for 40 min and the lysate was collected by centrifugation at 11,000 rpm for 20 min.

Purification of recombinant α1,3GalT

The enzyme was purified using a Ni^{2+}-NTA affinity column (Qiagen), which binds to the hexahistidine tag. Purification was performed at 4°C. The Ni^{2+} column was equilibrated with 3 volumes of 1× binding buffer (5 mM imidazole, 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl) before loading the cell lysate. The column was then washed exclusively with 6 volumes of 1× binding buffer, followed by 6 volumes of 1× washing buffer (60 mM imidazole, 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl)) and 6 volumes of 1× elution buffer (200 mM imidazole, 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl). After elution, the fractions containing the purified enzyme (detected by a UV spectrophotometer) were combined, and the purified enzyme was dialyzed at 4°C against 10% glycerol, 20 mM Tris-HCl, pH 7.9 buffer for enzyme activity assays and enzymatic reactions. Polymerization status and purity of the sample were determined by fast-performance liquid chromatography FPLC (AKTA FPLC, Amersham Pharmacia Biotech, Piscataway, NJ) carried out using a Superdex 200 prep column pre-equilibrated with 50 mM ammonium acetate buffer (pH 7.0) @ 0.5 mL/min (Figure 2).

α1,3-Galactosyltransferase assay

Enzyme activity for different acceptors was assayed at 37°C for 12 min in a final volume of 100 μL containing 10 mM Tris-HCl (pH 7.0), 10 mM $MnCl_2$, 0.1% BSA, 0.3 mM UDP-[6-^{3}H]Gal (final specific activity of 500 cpm/nmol), 0.04 mg/mL

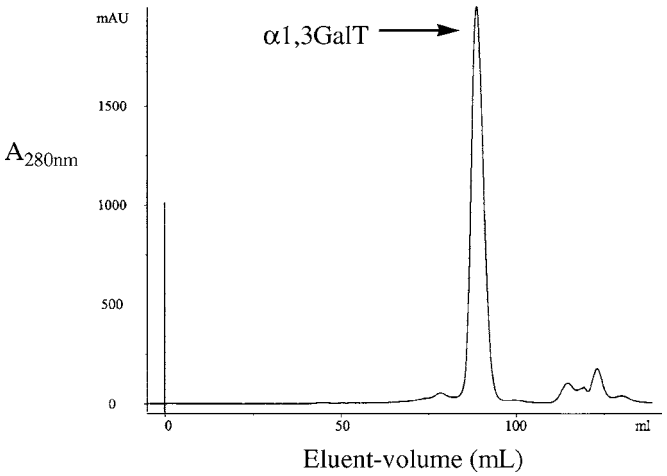


Figure 2. FPLC elution trace for IMAC purified α1,3GalT.

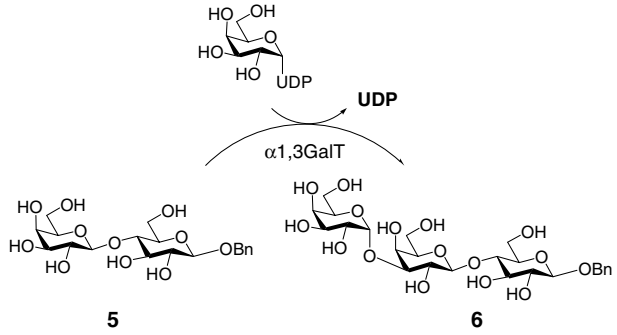
enzyme, and 50 mM acceptor. Acceptor was omitted for the blank. The reaction was stopped by adding 100 μL of ice-cold 0.1 M EDTA, and to the mixture was added Dowex 1 × 8–200 chloride anion exchange resin. The eluent was filtered and collected in a 20-mL plastic vial. ScintiVerse BD (5 mL, Fisher) was added, and the vial was vortexed thoroughly. The radioactivity of the enzyme was counted in a liquid scintillation counter (Beckmann LS-3801 counter). One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of galactose from UDP-Gal to lactose per minute at 37°C.

Acceptor specificity assay for α1,3GalT

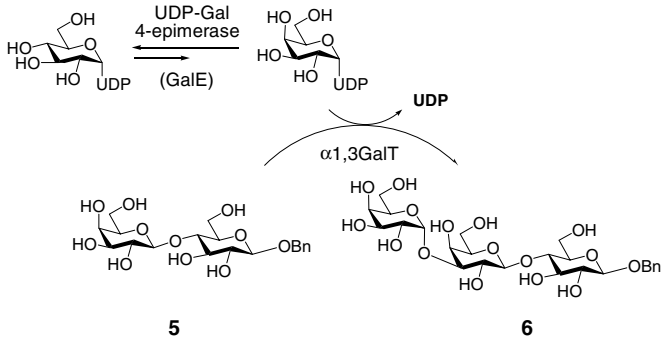
This assay was performed as described above except that the acceptor for α1,3GalT was varied and the reaction was carried out in 10 mM Tris/HCl buffer at pH 7.0. The acceptor has been excluded for the background count (Scheme 1).

General procedure for galactosylation of Gal$\beta(1 \rightarrow 4)$ Glcβ–OBn (**5**) and analogues using the dual enzyme system

To a mixture of the acceptor **5** (480 μmol, 40 mM), UDP-glucose (576 μmol, 48 mM), $MnCl_2$ (10 mM), and bovine



Scheme 1. Single enzyme glycosylation for the production of Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glcβ-OBn epitope **6**.



Scheme 2. UDP-Gal 4-epimerase (GalE) and α1,3GalT act in tandem to reduce the overall cost for the production of Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glcβ–OBn epitope **6**.

serum albumin (BSA) (0.1%) in Tris-HCl buffer (100 mM, pH 7.0, 12 mL) were added the enzymes UDP-galactose-4-epimerase (GalE) (10 U) and α1,3GalT (7 U) (Scheme 2). The reaction was shaken under an argon atmosphere at room temperature (ca. 25°C) for 3 days. The reaction was monitored by TLC [iPrOH/H_2O/NH_4OH = 7:3:2 (v/v/v)]. After 24 h, methanol was added to the reaction mixture to precipitate the proteins, which were subsequently removed by centrifugation followed by filtration through a 0.20 μm Fisherbrand nylon filter. The supernatant was then concentrated and loaded onto a G-15 gel filtration column (120 cm × 4 cm) with water as the eluent. The desired fraction was collected and lyophilized to yield the product. One unit (U) of GalE activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of UDP-Glc to UDP-Gal per min at 24°C [18].

Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glcβ–OBn **6** analysis

^{1}H and ^{13}C NMR (500 MHz) spectra were obtained using a 500-MHz Varian Unity spectrometer with the chemical shift expressed as parts per million downfield using deuterated water as solvent. The following data were obtained. ^{1}H NMR (D_2O standard): δ 7.33-7.24 (m, 3 H), 6.93 (d, J = 7.5 Hz, 2 H), 4.97 (d, J = 3.5 Hz, 1 H), 4.39 (d, J = 8.5 Hz, 1 H), 4.35 (d, J = 7.5 Hz, 1 H), 4.01 (t, J = 6.5 Hz, 1 H), 4.00 (d, J = 2.5 Hz, 1 H), 3.46-3.84 (m, 15 H), 3.25 (t, J = 9.0 Hz, 1 H); ^{13}C NMR (D_2O): δ 130.47, 128.91, 128.86, 128.63, 119.04, 114.86 102.96, 101.16, 95.55, 77.77, 77.03, 76.55, 74.94, 74.30, 70.71, 69.45, 69.16, 69.00, 68.08, 64.69, 60.90, 60.80, 58.84; MS NEG ESI found 592.96 $(M\text{-}H)^+$.

Isolation of polyclonal anti-αGal antibody from human serum

Polyclonal anti-αGal antibody was isolated from commercially available human male type AB serum using an αGal (immobilized trisaccharide [Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$GlcNAc] on sepharose beads) affinity chromatography column [19,20]. The human serum was heated at 56°C in a water bath in order to inactivate the human complement. After 30 min the serum was cooled to ambient temperature and passed through the column to allow for binding between the anti-αGal antibody and the immobilized αGal epitope. After extensive washing with phosphate buffered saline (PBS, pH 7.4), the bound anti-αGal antibody was eluted with glycine-HCl buffer (pH 2.8). The eluent containing the antibodies, as monitored by UV spectroscopy (280 nm), was immediately adjusted to pH 7.2 using 0.1 M NaOH. The resulting antibody solution was stored as frozen aliquots (about 200 μg/ml) in PBS buffer.

ELISA inhibition assay with mouse laminin

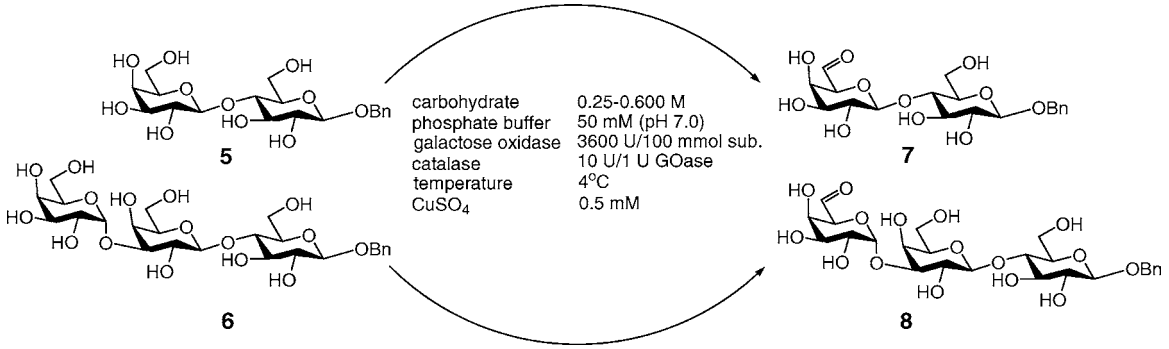
An ELISA assay was conducted using mouse laminin, as the solid-phase immobilized antigen. The purified human anti-αGal antibody was initially incubated, at varying concentrations of the αGal derivatives (**6**, **15–30**) for 2 h at room temperature with gentle shaking. An aliquote (50 μL) of the mixture was then added to each microtiter plate (Immulon 4) well which was pre-coated with mouse laminin (50 μL/well of 10 μg/ml in 0.1 N Na_2CO_3-$NaHCO_3$ buffer, pH = 9.5). After a 1.5 h incubation period at room temperature, unbound antibodies were washed out with PBS-tween (pH 7.4 + 0.2% tween). Next a secondary (1/1000 peroxidase conjugated goat anti-human IgG; 50 μL/well) antibody was introduced and allowed to incubate for approximately 1 h. After extensive washing using PBS-tween buffer solution, the substrate TMB (3, 3′5,5′-tetramethylbenzidine: H_2O_2, 9:1; 100 μL/well) was added. Enzymatic oxidation stained each well in varying intensities of blue; absorption was measured at 650 nm. The oxidation was quenched by the addition of 1 N H_2SO_4 (100 μL/well) and the optical absorptions were then measured at 450 nm. PBS with secondary antibody was used as background control. Purified anti-αGal antibody with secondary antibody was used as a positive control yielding the maximum intensity (0% inhibition). The % inhibition was calculated using the following equation:

$$(M - S) \div (M - B) = \%\ \text{inhibition}.$$

S refers to the OD_{450} reading of the sample at different concentrations of αGal derivatives. B is the OD_{450} reading of the background. M is the OD_{450} reading of the maximum staining. IC_{50} values were calculated from the % inhibition versus concentration plot of the inhibitors.

General procedure for enzymatic oxidation using galactose oxidase (EC 1.1.3.9, GAO)

In a typical experiment, a mixture of Gal$\beta(1 \rightarrow 4)$Glcβ–OBn (**5**) (1 g), 500 units of galactose oxidase (EC 1.1.3.9, GAO) and 6000 units of catalase in 40 mL phosphate buffer (50 mM pH 7.0) containing 6 mg of $CuSO_4 \cdot 5H_2O$ was stirred gently at 4°C with a continuous stream of air (air pump) (Scheme 3). After the starting material was completely oxidized, as indicated by DEPT NMR, the reaction mixture was heated at 100°C for 5 min to denature the enzymes. Centrifugation at 3200 rpm for 30 min followed by filtration through a 0.20 μm Fisherbrand



Scheme 3. Galactose Oxidase (EC 1.1.3.9, GAO) selectively oxidizes terminal galactose residues to aldehydes, as illustrated, in the conversion of Galβ(1 → 4)Glcβ–OBn (**5**) and Galα(1 → 3)Galβ(1 → 4)Glcβ–OBn (**6**) to compounds **7** and **8**.

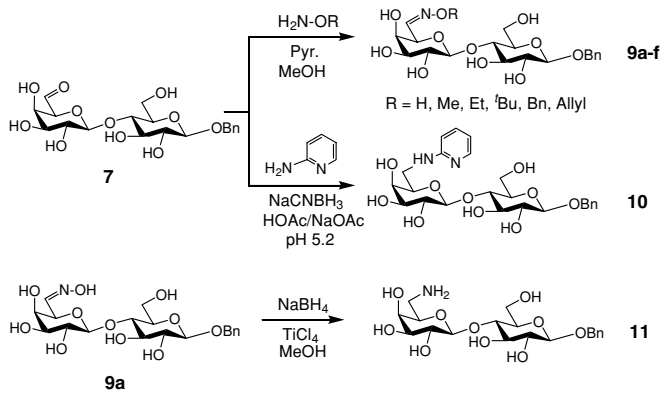
nylon filter gave the filtrate, which was lyophilized to produce a white solid **2** (990 mg) [21].

General procedure for the formation of oximes, reductive amination and formation of amines

Compound **7** (200 mg) was dissolved in 5 mL of dry methanol. Alkoxyamines (1 eq w/w) were added along with 0.2 mL of pyridine. The reaction was stirred for 2 h and then evaporated to dryness. Column chromatography, using a 17:3:1 (ethylacetate:methanol:water) eluent gave the purified product. R_f values varied for every newly formed oxime [22].

Compound **7** (100 mg) was dissolved in 5 mL of a 1 M NaOAc/HOAc buffer pH 5.2, then 2-aminopyridine (2 mol eq) was added along with 1.5 eq of $NaBH_3CN$ and the reaction was allowed to stir overnight. The workup consisted of lyophilization to dryness and then column chromatography (eluent as above) for purification to yield the desired compound.

(50 mg) of compound **9a** was dissolved in 10 mL dry methanol. $NaBH_4$ and $TiCl_4$ (3 eq each) were added and the reaction was stirred at rt for 8 h. The mixture was concentrated under reduced pressure and then purified using SEC (Scheme 4).



Scheme 4. Modification of the C-6′ position produces a small library of potential α1,3GalT substrates.

Results

Efficient utilization of UDP-donors

Although the activity assay for α1,3GalT was reportedly performed at pH 6.0 [23], our recombinant α1,3GalT displayed optimal activity at pH 7.0. At pH 6.0 in 50 mM MES (2[*N*-morpholino]ethanesulfonic acid) buffer, the recombinant α1,3GalT only has about 55% activity compared to that at pH 7.0 in 50 mM Tris/HCl buffer. There was only a 20% change in activity level within the range from pH 7.0 to 8.5, and an abrupt decrease in activity was observed when the pH value exceeded 8.5. We previously demonstrated that the optimal pH for GalE activity was also 7.0 in Tris/HCl buffer [18]. Therefore, the reactions catalyzed by two recombinant enzymes were carried out at pH 7.0 in 10 mM Tris/HCl buffer for complete optimization. It was imperative that optimal reaction conditions be determined on a μmol scale due to the relatively expensive UDP-Gal donor. The introduction of GalE decreases the overall cost of utilizing an α1,3GalT enzyme for the production of αGal analogues due to the fact that a less expensive UDP-Glc donor can be used as the logical substitute (refer to Schemes 1 and 2). It is important to note that enzymatic synthesis of a regio- and stereoselective $\alpha(1 \rightarrow 3)$ linkage is far more efficient than if the same compound were to be prepared chemically. Due to progress in recombinant DNA technology, the possiblility to express large quantities of glycosyltransferases that biosynthesize oligosaccharides has gathered considerable attention. The α1,3GalT transfers a single pyranosyl residue from UDP-Gal to a growing carbohydrate chain. Numerous examples have been reported in which glycosyltransferases tolerate structural changes on both donor and acceptor substrates, thus making enzymatic synthesis a promising alternative for the preparation of both natural and unnatural oligosaccharides [23–26].

Rational for utilizing galactose oxidase (EC 1.1.3.9, GAO)

Galactose oxidase (GAO) catalyzes the oxidation of many primary alcohols, alditols as well as the C-6 hydroxy group in D-galactose, D-talose, and D-gulose to the corresponding

aldehydes [27,28]. It is a convenient enzyme for synthetic applications because it can be easily obtained from a fungus and has good stability. GAO is very efficient in the oxidation of a broad range of galactosides, including oligo- and polysaccharides. It selectively oxidizes exposed primary hydroxyl groups in nonreducing, terminal galactose and *N*-acetyl galactosamine residues to the corresponding aldehyde (Scheme 3). The galactose oxidase/$NaBH_3CN$ method is well known and widely used as a technique to label cell-surface glycoconjugates [29,30]. The native reaction on galactose yields a C-6 aldehyde, which has been shown to undergo spontaneous Schiff base formation in the presence of amines [28]. With GAO we were able to oxidize 10 g of Gal$\beta(1 \rightarrow 4)$Glcβ–OBn (**5**) over a two-week period to the corresponding aldehyde in quantitative yield as determined by DEPT analysis, C-6′-OH 62–65 ppm which reappears as a hydrated aldehyde (peak at $\cong$ 88 ppm). The addition of catalase was extremely important as for every molecule of sugar oxidized, a molecule of hydrogen peroxide is produced. Catalase is known to convert hydrogen peroxide to water and molecular oxygen. Without catalase in the system, the byproduct (H_2O_2) would degrade the galactose oxidase enzyme. The Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glcβ–OBn trisaccharide (**6**), prepared by either the single or dual enzymatic transferase system, was also quantitatively oxidized to the corresponding C-6″ aldehyde. Not surprisingly, none of the C-6′-OH was oxidized in the process due to the fact that a terminal galactosyl residue is required because the C-4″-OH coordinates to the Cu^{II} containing metalo-protein [31].

Preparation of αGal analogues

The high specificity in the formation of glycosides by glycotransferases makes them a viable strategic choice for the preparative synthesis of complicated oligosaccharides and glycoconjugates. Prior to conducting substrate specificity assays of the transferase, analogues of αGal were prepared. A number of oxime analogues were prepared as described in the methods section. Oxime reduction was accomplished by using $NaBH_4$ with $TiCl_4$ in methanol to produce the C-6′-NH_2 analogue (**11**). Also reductive amination was successfully accomplished using 2-aminopyridine as the nucleophile and $NaBH_3CN$ in NaOAc/HOAc buffer pH 5.2 as the reductant to give rise to **10**. It is important that mild alkaline conditions be used in the oxime synthesis; under strong basic conditions β-elimination would occur. The same set of reactions were conducted on the Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glcβ–OBn trisaccharide (**6**) once the GAO reaction was complete.

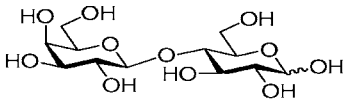
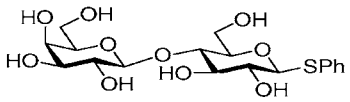
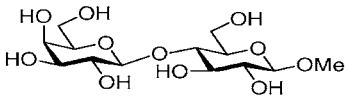
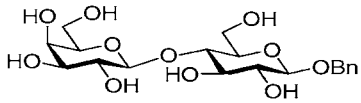
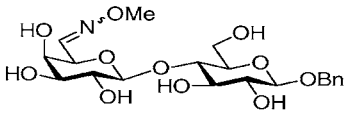
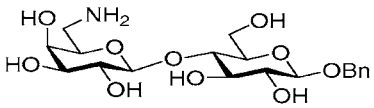
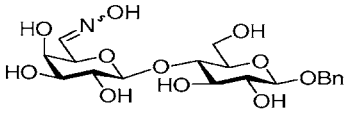
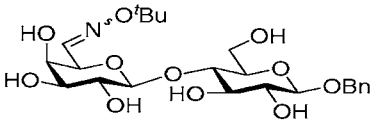
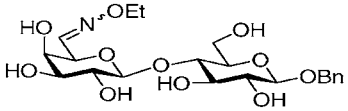
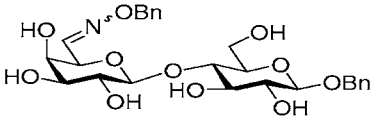
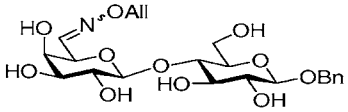
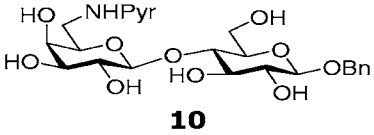
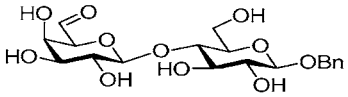
Sustrate specificity assay

α1,3GalT, which is absent in humans, catalyzes the formation of Gal$\alpha(1 \rightarrow 3)$Gal epitopes, the major xenoactive antigen which causes hyperacute rejection during xenotransplantation. The enzyme can use both Gal$\beta(1 \rightarrow 4)$GlcNAcβ-OR and Gal$\beta(1 \rightarrow 4)$Glcβ-OR as acceptors. Acceptor hydroxyl group mapping studies showed that Gal C-4′-OH is, according to the terminology, a key polar group essential for substrate recognition [32]. Substitution of Gal C-3′-OH with an amino group produces an inhibitor for this enzyme. The results suggested that C-3′-OH is not essential for binding to the enzyme, although it is the site of glycosylation (Table 1). Surprisingly, all of the αGal analogues produced were found to be substrates in the radioactive Sep-Pak assay.

It is quite remarkable that the enzyme can tolerate the introduction of large substituents at the C-6′ position, even though it is required for recognition, albeit not as strong an effect as the C-4′-OH. However it has been revealed by kinetic data, that steric increase does make it more difficult for the enzyme to bind and transfer. Thus in our study of substrate specificity, the analogues were being turned over but at a slower rate than the parent compound.

Once the substrate activity assays were accomplished, a directed effort to produce the αGal analogues was put forth. These compounds were prepared much in the same conditions as the assay, except for a greater quantity of substrate was used and the non-tritiated UDP donor was used instead of UDP-[6-^{3}H]Gal. All newly formed trisacchares were purified using a SEC column. Large scale production of the Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Glcβ–OBn trisaccharide (**6**) compound was necessary so as to produce quantities sufficient for the chemoenzymatic synthesis of compounds **23–30** for inhibition ELISA assays against the anti-αGal antibody. The ELISA assay was conducted to probe the antibody recognition of αGal epitope analogues. The main objective was to find an analogue that would bind with a higher affinity than those naturally found in the hopes of developing a strategy for antagonizing anti-αGal antibody in the human system. The ELISA provided direct evidence for the binding specificity of the varying epitopes **6**, **15–30**. As illustrated in Table 2, those epitopes modified on the C-6′ position were recognized by the IgG antibody in the 2–40 μM range of IC_{50} inhibition. It is important to note that the size of the substituent on the C-6′ position did not have a detrimental effect on the overall binding affinity. This leads to the conclusion that although the C-6' position of the αGal epitope plays a role in the overall binding affinity to the antibody, it is not imperative for binding. As illustrated in Table 2, it can be modified with hydrophilic, hydrophobic and aromatic groups and the antibody will still recognize it as an epitope. Entry 9 in Table 2 (**22**), however, illustrates that the antibody is very selective against charged functional groups because in the pH 7 range we assume the primary amine is in its charged form. In the cases where the C-6″ position was modified with the same oxime and reductive amination protocols as in entries 2–9 Table 2 (**15–22**), the severity of modification was directly evident. Aside from compounds **22**, **27–30** showing no inhibition, those that did give an IC_{50} value, were 15 to 40 times fold less potent than their counterparts modified at the C-6′ position.

Table 1. Acceptor specificity of α1,3GalT

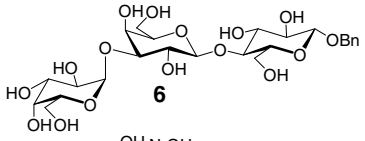
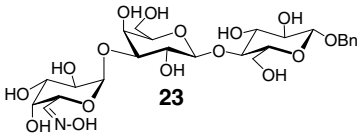
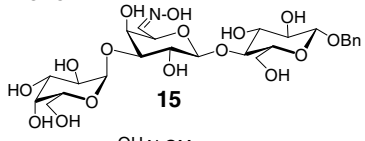
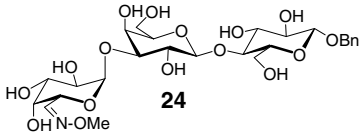
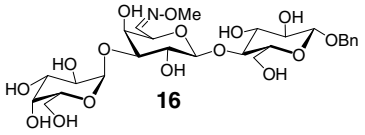
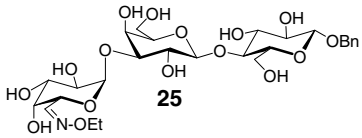
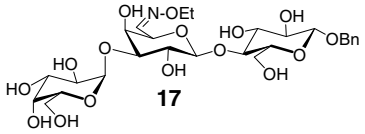
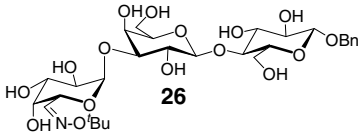
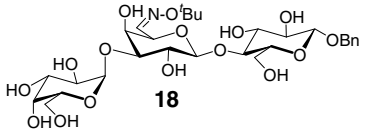
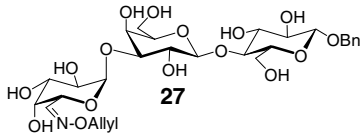
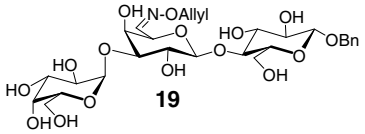
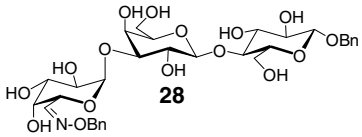
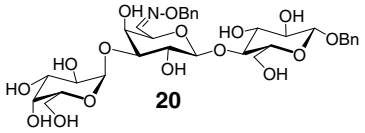
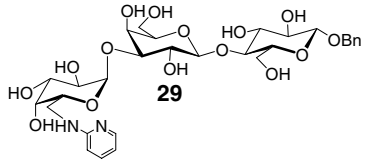
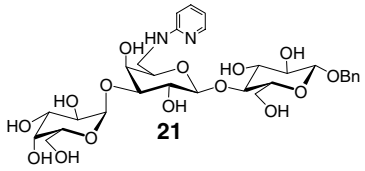
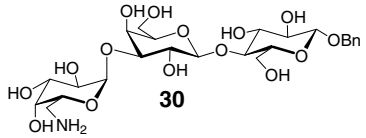
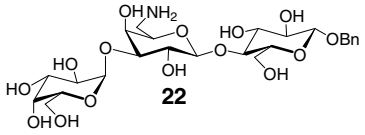
Entry	*Acceptor compound*	*Relative activity (%)*	*Entry*	*Acceptor compound*	*Relative activity (%)*
	Galβ(1→4)Glc derivatives				
1	 **12**	100	**3**	 **14**	94
2	 **13**	117	**4**	 **5**	121
	C-6′- Modified Galβ(1→4)Glc derivatives				
5	 **9b**	146	**10**	 **11**	85
6	 **9a**	137	**11**	 **9d**	67
7	 **9c**	123	**12**	 **9e**	65
8	 **9f**	100	**13**	 **10**	60
9	 **7**	85			

Spectral data

Compound **15** *Methyl* α-D-*galactopyranosyl-(1 → 3)-β*-D-*galactopyranosyl-C-6-O-H oxime-(1 → 4)-β*-D-*glucopyranoside OBn*: ^{1}H NMR (400 MHz, D_2O): δ 7.56 (d, $J = 4.8$ Hz, 2 H), 7.43, (m, 3 H), 6.90 (d, $J = 1$ Hz, 1 H), 5.15 (d, 1 H, $J =$ 4.1 Hz, 1 H), 4.95 (d, $J = 12$ Hz, 1 H), 4.35 (d, $J = 8.1$ Hz, 1 H), 4.25 (d, $J = 8.1$ Hz, 1 H), 4.20 (t, $J = 6.6$ Hz, 1 H), 3.40–3.89 (m, 16 H), 3.14 (t, $J = 8.6$ Hz, 1H). ^{13}C NMR (100 MHz, D_2O): δ 148.88, 137.01, 129.15, 128.88, 103.42, 101.18, 95.98, 78.78, 77.49, 75.58, 74.96, 74.60, 73.02, 72.30, 71.05, 70.96, 69.27, 69.08, 68.71, 60.64, 60.52, 60.15; HRFABMS: calcd for $C_{25}H_{37}NO_{16}Na$ ($M + Na^+$) 630.2112; found 630.2136.

Compound **16** α-D-*galactopyranosyl-(1 → 3)-β*-D-*galactopyranosyl-C-6-O-Me-oxime-(1 → 4)-β*-D-*glucopyranoside OBn*: ^{1}H NMR (400 MHz, D_2O): δ 7.48 (d, $J = 5.6$ Hz, 0.75 H), 7.41–7.24 (m, 5 H), 6.80 (d, $J = 4.8$ Hz, 0.25 H), 4.88 (d, $J = 3.0$ Hz, 1 H), 4.76 (d, $J = 7.6$ Hz, 1 H), 4.32 (d, $J = 7.6$ Hz, 1 H), 4.17 (t, $J = 6.1$ Hz, 1 H), 3.85 (m, 2 H), 3.81–3.40 (m, 15 H), 3.38 (s, 3 H), 3.34 (m, 6 H); ^{13}C NMR (100 MHz, D_2O): δ 147.04, 137.75, 128.24, 128.08, 127.68, 103.33, 101.92, 95.65, 79.29, 78.88, 78.29, 77.45, 75.95, 75.55, 72.25, 71.66, 71.00, 70.92, 69.25, 69.04, 68.68, 62.58, 60.60, 60.50, 60.17; HRFABMS: calcd for $C_{26}H_{39}NO_{16}Na$ ($M + Na^+$) 644.2269; found 644.2169.

Table 2. ELISA inhibition results for αGal epitope/analogues

Entry	*Compound*	*IC_{50} values (μM) IgG*	*Entry*	*Compound*	*IC_{50} values (μM) IgG*
1	 **6**	3	10	 **23**	47
2	 **15**	3	11	 **24**	84
3	 **16**	2	12	 **25**	>100
4	 **17**	10	13	 **26**	>100
5	 **18**	30	14	 **27**	N.I.
6	 **19**	7	15	 **28**	N.I.
7	 **20**	37	16	 **29**	N.I.
8	 **21**	20	17	 **30**	N.I.
9	 **22**	N.I.			

Compound **17** α-D-*galactopyranosyl-(1 → 3)-β*-D-*galactopyranosyl-C-6-O-Et-oxime-(1 → 4)-β*-D-*glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.48 (d, J = 5.6 Hz, 0.66 H), 7.41–7.24 (m, 5 H), 6.76 (d, J = 4.8 Hz, 0.34 H), 4.67 (d, J = 11.0 Hz, 1H), 4.44 (d, J = 14.0 Hz, 1 H), 4.32 (d, J = 15.0 Hz, 1 H), 4.17 (t, J = 12.0 Hz, 1 H), 3.72 (m, 2 H), 3.69–3.50 (m, 15 H), 3.40 (m, 2 H), 3.34 (t, J = 8 Hz, 1 H), 1.22 (m, 3 H); ^{13}C NMR (100 MHz, D_2O): δ 148.27, 136.75, 129.01, 128.97, 128.75, 103.21, 101.25, 94.57, 79.29, 78.88, 78.29, 76.18, 74.98, 74.60, 74.46, 73.13, 73.07, 72.63, 72.31, 71.77, 70.85, 70.66, 70.59, 70.32, 69.93, 68.92, 60.23, 13.82; HRFABMS: calcd for $C_{27}H_{41}NO_{16}Na$ (M + Na^+) 658.2425; found 658.2430.

Compound **18** α-D-*galactopyranosyl-(1 → 3)-β*-D-*galactopyranosyl-C-6-O-t-Bu-oxime-(1 → 4)-β*-D-*glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.43 (d, J = 8.0 Hz, 0.60 H), 7.34–7.26 (m, 5 H), 6.75 (d, J = 4.5 Hz, 0.40 H), 4.68 (d, J = 11.5 Hz, 1 H), 4.45 (d, J = 7.0 Hz, 1 H), 4.38 (d, J =

8.0 Hz, 1 H), 4.25 (d, $J = 6.0$ Hz, 1 H), 4.13 (d, $J = 2.5$ Hz, 1 H), 3.95–3.84 (m, 14 H), 3.62–3.50 (m, 6 H), 3.40 (m, 2 H), 1.28 (s, 4 H), 1.26 (s, 5 H); ^{13}C NMR (125 MHz, D_2O): δ 146.92, 145.75, 137.83, 128.10, 127.98, 127.53, 103.87, 102.01, 96.45, 79.58, 78.99, 78.68, 75.29, 75.17, 74.98, 74.60, 74.46, 73.61, 73.51, 73.21, 71.29, 70.98, 70.84, 70.65, 70.40, 69.30, 60.72, 26.61; HRFABMS: calcd for $C_{29}H_{45}NO_{16}Na$ ($M + Na^+$) 686.2738; found 686.2742.

Compound **19** *α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl-C-6-O-Allyl-oxime-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.48 (d, $J = 5.6$ Hz, 0.50 H), 7.42–7.24 (m, 5 H), 6.82 (d, $J = 4.8$ Hz, 0.20 H), 6.01–5.92 (m, 2 H), 5.27 (d, $J = 18$ Hz, 1 H), 5.18 (t, $J =$ 8.8 Hz, 1 H), 4.94–4.9 (m, 1 H), 4.67 (d, $J = 12.4$ Hz, 1 H), 4.48 (d, $J = 16.2$ Hz, 1 H), 4.32 (d, $J = 17.3$ Hz, 1 H), 4.17 (t, $J = 16.2$ Hz, 1 H), 3.72 (m, 2 H), 3.69–3.50 (m, 15 H), 3.40 (m, 2 H), 3.34 (t, $J = 9.7$ Hz, 1 H), ^{13}C NMR (100 MHz, D_2O): δ 147.24, 137.81, 134.13, 128.15, 128.01, 127.56, 116.88, 103.80, 101.96, 96.13, 79.32, 78.88, 78.29, 76.18, 75.27, 75.13, 75.06, 74.98, 74.60, 74.46, 73.58, 73.13, 73.07, 72.63, 72.31, 71.77, 70.85, 70.66, 70.59, 70.32, 69.93, 68.92, 60.23; HRFABMS: calcd for $C_{28}H_{41}NO_{16}Na$ ($M + Na^+$) 670.2425; found 670.2441.

Compound **20** *α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl-C-6-O-Bn-oxime-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.54 (d, $J = 5.5$ Hz, 0.4 H), 7.42 (d, $J = 8.0$ Hz, 1.0 H), 7.35–7.26 (m, 4 H), 6.84 (d, $J =$ 4.0 Hz, 0.2 H), 5.11 (d, $J = 3.0$ Hz, 1 H), 5.07 (s, 2 H), 4.67 (d, $J = 11.5$ Hz, 1 H), 4.44 (d, $J = 7.0$ Hz, 1 H), 4.38 (d, $J = 8.0$ Hz, 1 H), 4.24 (d, $J = 6.0$ Hz, 1 H), 3.94 (m, 3 H), 3.69–3.50 (m, 14 H), 3.63–3.52 (m, 2 H), 3.42–3.34 (m, 2 H); ^{13}C NMR (125 MHz, D_2O): δ 148.83, 147.51, 137.82, 137.70, 128.32, 128.26, 128.24, 128.17, 128.13, 128.02, 127.88, 127.78, 127.59, 103.36, 102.00, 95.57, 79.34, 78.91, 76.43, 76.05, 75.28, 75.13, 73.61, 73.13, 73.08, 72.63, 72.31, 71.77, 70.85, 70.66, 70.59, 70.32, 69.93, 68.92, 60.63; HRFABMS: calcd for $C_{32}H_{43}NO_{16}Na$ ($M + Na^+$) 720.2582; found 720.2570.

Compound **21** *α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl-C-6-2-aminopyridine-(1→4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.84 (d, $J = 5.5$ Hz, 2 H), 7.56 (t, $J = 8.0$ Hz, 1 H), 7.41 (d, $J = 7.0$ Hz, 2 H), 7.32 (t, J = 7.5 Hz, 2 H), 7.27 (d, $J = 7.0$ Hz, 1 H), 6.8 (d, $J = 8.5$ Hz, 2 H), 6.64 (t, $J = 6.5$ Hz, 1 H), 4.67 (d, $J =$ 11.5 Hz, 2 H), 4.38 (dd, $J = 2.5$, 8.0 Hz, 3 H), 3.94 (m, 3 H), 3.69–3.50 (m, 14 H), 3.63–3.52 (m, 2 H), 3.42–3.34 (m, 2 H); ^{13}C NMR (125 MHz, D_2O): δ 139.68, 137.79, 128.13, 128.02, 127.57, 112.62, 110.32, 103.94, 101.94, 95.32, 79.79, 78.91, 76.43, 76.05, 75.90, 75.35, 75.18, 75.13, 73.58, 73.13, 73.08, 72.63, 72.31, 71.77, 71.34, 70.85, 70.64, 69.14, 61.34, 60.63; HRFABMS: calcd for $C_{30}H_{42}N_2O_{15}Na$ ($M + Na^+$) 693.2585; found 693.2594.

Compound **22** *α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl-C-6-amino-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (300 MHz, D_2O): δ 7.42 (d, $J = 6.6$ Hz, 2 H), 7.35–7.26 (m, 3 H), 4.65 (d, $J = 6.0$ Hz, 2 H), 4.37 (t, $J = 6.6$ Hz, 3 H), 3.92 (m, 3 H), 3.81–3.70 (m, 2 H), 3.60–3.48 (m, 15 H), 3.31 (q, $J = 1.5$ Hz, 4 H); ^{13}C NMR (75 MHz, D_2O): δ 130.47, 128.91, 128.86, 128.63, 119.04, 114.86, 103.41, 101.37, 78.82, 78.71, 77.51, 75.58, 74.99, 74.56, 74.04, 72.30, 71.59, 71.35, 71.06, 70.96, 70.30, 69.28, 69.08, 68.71, 60.65, 60.53, 60.18; HRFABMS: calcd for $C_{25}H_{39}NO_{15}Na$ ($M + Na^+$) 616.2320; found 617.2413.

Compound **23** *Methyl α-D-galactopyranosyl-C-6-O-H oxime-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.47 (d, $J =$ 6.0 Hz, 0.75 H), 7.41, (m, 5 H), 6.90 (d, $J = 3.0$ Hz, 0.25 H), 5.15 (d, $J = 4.0$ Hz, 1 H), 4.91 (d, $J = 12.0$ Hz, 1 H), 4.38 (d, $J = 8.0$ Hz, 1 H), 4.24 (d, $J = 8.0$ Hz, 1 H), 4.22 (t, $J = 7.0$ Hz, 1 H), 3.46–3.95 (m, 16 H), 3.10 (t, $J = 9.0$ Hz, 1 H). ^{13}C NMR (125 MHz, D_2O): δ 149.82, 139.01, 127.12, 126.82, 103.42, 101.18, 95.98, 78.78, 77.49, 75.58, 75.15, 74.96, 74.60, 73.02, 72.30, 71.89, 71.05, 70.96, 69.27, 69.08, 68.71, 60.64, 60.52, 60.15; FABMS: calcd for $C_{25}H_{37}NO_{16}Na$ ($M + Na^+$) 630.2112; found 630.

Compound **24** *α-D-galactopyranosyl-C-6-O-Me-oxime-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (400 MHz, D_2O): δ 7.48 (d, $J = 5.6$ Hz, 0.75 H), 7.41–7.24 (m, 5 H), 6.80 (d, $J = 4.8$ Hz, 0.25 H), 4.88 (d, $J = 3.0$ Hz, 1 H), 4.76 (d, $J = 7.6$ Hz, 1 H), 4.32 (d, $J = 7.6$ Hz, 1 H), 4.17 (t, $J = 6.1$ Hz, 1 H), 3.85 (m, 2 H), 3.81–3.40 (m, 15 H), 3.38 (s, 3 H), 3.34 (m, 6 H); ^{13}C NMR (100 MHz, D_2O): δ 147.04, 137.75, 128.24, 128.08, 127.68, 103.33, 101.92, 95.65, 79.29, 78.88, 78.29, 77.45, 75.95, 75.55, 72.25, 71.66, 71.00, 70.92, 69.25, 69.04, 68.68, 62.58, 60.60, 60.50, 60.17; FABMS: calcd for $C_{26}H_{39}NO_{16}Na$ ($M +$ Na^+) 644.2269; found 644.

Compound **25** *α-D-galactopyranosyl-C-6-O-Et-oxime-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.48 (d, $J = 5.6$ Hz, 0.66 H), 7.41–7.24 (m, 5 H), 6.76 (d, $J = 4.8$ Hz, 0.34 H), 4.67 (d, $J = 11.0$ Hz, 1 H), 4.44 (d, $J = 14.0$ Hz, 1 H), 4.32 (d, $J = 15.0$ Hz, 1 H), 4.17 (t, $J = 12.0$ Hz, 1 H), 3.72 (m, 2 H), 3.69–3.50 (m, 15 H), 3.40 (m, 2 H), 3.34 (t, $J = 8$ Hz, 1 H), 1.22 (m, 3 H); ^{13}C NMR (100 MHz, D_2O): δ 148.27, 136.75, 129.01, 128.97, 128.75, 103.21, 101.25, 94.57, 79.29, 78.88, 78.29, 76.18, 74.60, 74.46, 73.13, 73.07, 72.63, 72.31, 71.77, 70.85, 70.59, 70.32, 69.93, 68.92, 60.23, 13.82; FABMS: calcd for $C_{27}H_{41}NO_{16}Na$ ($M + Na^+$) 658.2425; found 658.47.

Compound **26** *α-D-galactopyranosyl-C-6-O-t-Bu-oxime-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.43 (d, $J = 8.0$ Hz, 0.60 H), 7.34–7.26 (m, 5 H), 6.75 (d, $J = 4.5$ Hz, 0.40 H), 4.68 (d, $J = 11.5$ Hz, 1 H), 4.45 (d, $J = 7.0$ Hz, 1 H), 4.38 (d, $J =$ 8.0 Hz, 1 H), 4.25 (d, $J = 6.0$ Hz, 1 H), 4.13 (d, $J = 2.5$ Hz, 1 H), 3.95–3.84 (m, 14 H), 3.62–3.50 (m, 6 H), 3.40 (m, 2 H), 1.28 (s, 4 H), 1.26 (s, 5 H); ^{13}C NMR (125 MHz, D_2O): δ 146.92, 145.75, 137.83, 128.10, 127.98, 127.53, 103.87,

102.01, 96.45, 79.58, 78.99, 78.68, 75.29, 75.17, 74.98, 74.60, 74.46, 73.61, 73.51, 73.21, 71.29, 70.98, 70.84, 70.65, 70.40, 69.30, 60.72, 26.61; FABMS: calcd for $C_{29}H_{45}NO_{16}Na$ ($M + Na^+$) 686.2738; found 686.21.

Compound **27** *α-D-galactopyranosyl-C-6-O-Allyl-oxime-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.48 (d, $J = 5.6$ Hz, 0.50 H), 7.42–7.24 (m, 5 H), 6.82 (d, $J = 4.8$ Hz, 0.20 H), 6.01–5.92 (m, 2 H), 5.27 (d, $J = 18$ Hz, 1 H), 5.18 (t, $J = 8.8$ Hz, 1 H), 4.94–4.9 (m, 1 H), 4.67 (d, $J = 12.4$ Hz, 1 H), 4.48 (d, $J = 16.2$ Hz, 1 H), 4.32 (d, $J = 17.3$ Hz, 1 H), 4.17 (t, $J = 16.2$ Hz, 1 H), 3.72 (m, 2 H), 3.69–3.50 (m, 15 H), 3.40 (m, 2 H), 3.34 (t, $J = 9.7$ Hz, 1 H), ^{13}C NMR (100 MHz, D_2O): δ 147.24, 137.81, 134.13, 128.15, 128.01, 127.56, 116.88, 103.80, 101.96, 96.13, 79.32, 78.88, 78.29, 76.18, 75.27, 75.13, 74.98, 74.60, 73.58, 73.13, 72.63, 72.31, 71.77, 70.66, 70.59, 69.93, 68.92, 60.23; FABMS: calcd for $C_{28}H_{41}NO_{16}Na$ ($M + Na^+$) 670.2425; found 670.

Compound **28** *α-D-galactopyranosyl-C-6-O-Bn-oxime-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.45 (d, $J = 6.0$ Hz, 0.6 H), 7.42 (d, $J = 8.0$ Hz, 1 H), 7.35–7.26 (m, 4 H), 6.84 (d, $J = 4.0$ Hz, 0.2 H), 5.11 (d, $J = 3.0$ Hz, 1 H), 5.07 (s, 2 H), 4.67 (d, $J = 11.0$ Hz, 1 H), 4.44 (d, $J = 7.0$ Hz, 1 H), 4.38 (d, $J = 8.0$ Hz, 1 H), 4.24 (d, $J = 6.0$ Hz, 1 H), 3.94 (m, 3 H), 3.69–3.50 (m, 14 H), 3.63–3.52 (m, 2 H), 3.42–3.34 (m, 2 H); ^{13}C NMR (125 MHz, D_2O): δ 148.83, 147.51, 137.82, 137.70, 128.32, 128.26, 128.24, 128.17, 128.13, 128.02, 127.88, 127.78, 127.59, 103.36, 102.00, 95.57, 79.34, 78.91, 76.43, 76.05, 75.28, 75.13, 73.61, 72.63, 72.31, 71.77, 70.85, 70.66, 69.93, 68.92, 60.63; FABMS: calcd for $C_{32}H_{43}NO_{16}Na$ ($M + Na^+$) 720.2582; found 721.

Compound **29** *α-D-galactopyranosyl-C-6-2-aminopyridine-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (500 MHz, D_2O): δ 7.84 (d, $J = 5.5$ Hz, 2 H), 7.56 (t, $J = 8.0$ Hz, 1 H), 7.41 (d, $J = 7.0$ Hz, 2 H), 7.32 (t, $J = 7.5$ Hz, 2 H), 7.27 (d, $J = 7.0$ Hz, 1 H), 6.8 (d, $J = 8.5$ Hz, 2 H), 6.64 (t, $J = 6.5$ Hz, 1 H), 4.67 (d, $J = 11.5$ Hz, 2 H), 4.38 (dd, $J = 2.5$, 8.0 Hz, 3 H), 3.94 (m, 3 H), 3.69–3.50 (m, 14 H), 3.63–3.52 (m, 2 H), 3.42–3.34 (m, 2 H); ^{13}C NMR (125 MHz, D_2O): δ 139.68, 137.79, 128.13, 128.02, 127.57, 112.62, 110.32, 103.94, 101.94, 95.32, 79.79, 78.91, 76.43, 76.05, 75.90, 75.35, 75.18, 75.13, 73.58, 73.13, 73.08, 72.63, 72.31, 71.77, 71.34, 70.85, 70.64, 69.14, 61.34, 60.63; FABMS: calcd for $C_{30}H_{42}N_2O_{15}Na$ ($M + Na^+$) 693.2585; found 693.57.

Compound **30** *α-D-galactopyranosyl-C-6-amino-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside OBn*: ^{1}H NMR (300 MHz, D_2O): δ 7.42 (d, $J = 6.6$ Hz, 2 H), 7.35–7.26 (m, 3 H), 4.65 (d, $J = 6.0$ Hz, 2 H), 4.37 (t, $J = 6.6$ Hz, 3 H), 3.92 (m, 3 H), 3.81–3.70 (m, 2 H), 3.60–3.48 (m, 15 H), 3.31 (q, $J = 1.5$ Hz, 4 H); ^{13}C NMR (75 MHz, D_2O): δ 130.47, 128.91, 128.86, 128.63, 119.04, 114.86, 103.41, 101.37, 78.82, 78.71, 77.51, 75.58, 74.99, 74.56, 71.59, 71.35, 71.06, 70.96, 70.30, 69.28, 69.08, 68.71, 60.65, 60.53, 60.18; FABMS: calcd for $C_{25}H_{39}NO_{15}Na$ ($M + Na^+$) 616.2320; found 617.

Anti-αGal antibody binding studies

Anti-αGal is the only known natural antibody that is found in large quantities in all humans who are not immunocompromised. It is a polyclonal antibody that constitutes approximately 1% of circulating immunoglobulins and is primarily of the IgG isotype. Anti-αGal is also present in the serum as IgM or IgA isotype antibodies. It interacts specifically with mammalian carbohydrate structures containing a terminal Galα(1 → 3)Gal (termed the αgalactosyl epitope). Not surprisingly, it does not cross react with other known mammalian carbohydrate epitopes, or with proteins lacking the αgalactosyl epitope. Anti-αGal does not bind to glycolipids that have terminal βgalactosyl, sialic acid, fucose, or N-acetyl lactosamine carbohydrate units. It is produced in humans throughout life as a result of continued antigenic stimulation by environmental antigens including gastrointestinal bacteria. The highly restricted specificity of anti-αGal and its over-abundant production in humans indicate that this natural antibody belongs to a different category from polyreactive natural antibodies, which react with various antigens and are present in small quantities in the circulatory system. The anti-αGal antibody binds to the epitope with an inhibition in the μmol range, which potentially opens opportunity for studies to increase the binding affinity. The analogues synthesized not only access the substrate specificity of the α1,3GalT, but also allowed us to examine their binding potential to the anti-αGal antibody (Table 2). The conformationally bound state of the epitope is currently not known and, therefore, it is also unknown which hydroxyl groups are involved in binding. What is known, however, is that the Galα(1 → 3)Gal link is critical for recognition. The results conclude that the C-6′ position of the αGal trisaccharide does not alter the binding affinity significantly. However, αGal trisaccharide analogues (**25–30**) did not lead to any significant binding which proves the C-6″ position is critical for anti-αGal antibody binding.

Discussion

We have successfully modified the αGal epitope utilizing environmentally benign conditions, namely the reaction of galactose oxidase (EC 1.1.3.9, GAO) coupled with dehydrative coupling of alkoxyamines to produce oximes as well as aqueous reductive amination. The analogues were prepared in an attempt to probe both the α1,3GalT and the anti-αGal antibody binding sites. It was discovered that modification of the C-6′-OH of Galβ(1 → 4)Glcβ–OBn did not significantly alter the ability of the transferase enzyme to recognize those compounds as substrates. The overall activity of the enzyme did decrease when the C-6′-OH was substituted with a fairly bulky group such as the case with compounds **9d**, **9e**, and **10** (entries 11–13 Table 1), however not to the point where the actual αGal epitope could not

be isolated over an extended reaction concorse. Interestingly, compound **11** was nicely recognized by the transferase. It was initially believed that the free amino group would hinder the compounds ability to act as a substrate due to the possibility of a charged species that could potentially lead to an unfavorable electronic effect. The interesting outcome to this probing study is that there exists a potential to modify the C-6′ position of the αGal epitope with fluorescence markers or biotinylated linkers to further examine the binding interaction of the antibody and epitope.

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References

1 Varki A, Biological roles of oligosaccharides: All of the theories are correct, *Glycobiology* **3**, 97–130 (1993).
2 Sharon N, Lis H, Carbohydrates in cell recognition, *Sci Am* **268**, 82–9 (1993).
3 Ellington AD, Szostak JW, *In vitro* selection of RNA molecules that bind specific ligands, *Nature* **346**, 818–22 (1990).
4 Tuerk C, Gold L, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, *Science* **249**, 505–10 (1990).
5 Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ, A new type of synthetic peptide library for identifying ligand-binding activity, *Nature* **354**, 82–4 (1991).
6 Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, *Nature* **355**, 564–6 (1992).
7 Toshima K, Tatsuta K, Recent progress in O-glycosylation methods and its application to natural products synthesis, *Chem Rev* **93**, 1503–31 (1993).
8 Barresi F, Hindsgaul O, Chemically synthesized oligosaccharides, 1994. A searchable table of glycosidic linkages, *J Carbohydr Chem* **14**, 1043–87 (1995).
9 Karlsson-Parra A, Ridderstad A, Wallgren AC, Moller E, Ljunggren HG, Korsgren O, Xenograft rejection of porcine islet-like cell clusters in normal and natural killer cell-depleted mice, *Transplantation* **61**, 1313–20 (1996).
10 McKane W, Lee J, Preston R, Hacking A, Simpson P, Lynds S, Goldberg L, Cairns T, Taube D, Polymorphism in the human anti-pig natural antibody repertoire: Implications for antigen-specific immunoadsorption, *Transplantation* **66**, 626–33 (1998).
11 Wang L, Radic MZ, Galili U, Human anti-Gal heavy chain genes. Preferential use of VH3 and the presence of somatic mutations, *J Immun* **155**, 1276–85 (1995).
12 Galili U, Andrews P, Suppression of a-galactosyl epitope synthesis and production of the natural anti-Gal antibody: A major evolutionary event in ancestral Old World primates, *J Human Evol* **29**, 433–42 (1993).
13 McAuliffe JC, Hindsgaul O, Carbohydrate drugs-an ongoing challenge, *Chem & Ind* **5**, 170–4 (1997).
14 Chen X, Zhang W, Wang J, Fang J, Wang PG, Production of alpha-galactosyl epitopes via combined use of two recombinant whole cells harboring UDP-galactose 4-epimerase and alpha-1,3-galactosyltransferase, *Biotech Prog* **16**, 595–99 (2000).
15 Zhang J, Kowal P, Fang J, Andreana P, Wang PG, Efficient chemoenzymatic synthesis of globotriose and its derivatives with a recombinant α(1,4)-galactosyltransferase, *Carb Res* **337**, 969–76 (2002).
16 Zhang W, Wang J-Q, Li J, Yu L-B, Wang PG, Large-Scale Synthesis of an αGalactosyl Trisaccharide Epitope Involved in the Hyperacute Rejection Upon Xenotransplantation, *J Carbohydr Chem* **18**, 1009–17 (1999).
17 Fang J, Li J, Chen X, Zhang Y, Wang J, Guo Z, Zhang W, Yu L, Brew K, Wang PG, Highly efficient chemoenzymic synthesis of α-galactosyl epitopes with a recombinant $\alpha(1 \rightarrow 3)$-galactosyltransferase, *J Am Chem Soc* **120**, 6635–8 (1998).
18 Chen X, Kowal P, Hamad S, Fan H, Wang PG, Cloning, expression and characterization of a UDP-galactose 4-epimerase from *Escherichia coli*, *Biotech Lett* **21**, 1131–5 (1999).
19 Xu Y, Lorf T, Sablinski T, Gianello P, Bailin M, Monroy R, Kozlowski T, Awwad M, Cooper DKC, Sachs DH, Removal of anti-porcine natural antibodies from human and nonhuman primate plasma *in vitro* and *in vivo* by a Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$Gleβ-X immunoaffinity column, *Transplantation* **65**, 172–9 (1998).
20 Taniguchi S, Neethling FA, Korchagina EY, Bovin N, Ye Y, Kobayashi T, Niekrasz M, Li S, Koren E, *In vivo* immunoadsorption of antipig antibodies in baboons using a specific Gal$\alpha(1 \rightarrow 3)$Gal column, *Transplantation* **62**, 1379–84 (1996).
21 Andreana PR, Xie W, Cheng HN, Qiao L, Murphy DJ, Gu Q-M, Wang PG, *In Situ* Preparation of β-D-1-O-Hydroxylamino Carbohydrate Polymers Mediated by Galactose Oxidase, *Org Lett* **4**, 1863–6 (2002).
22 Andreana PR, Sanders T, Janczuk A, Warrick JI, Wang PG, Chemoenzymatic synthesis of polyhydroxyazepanes, *Tetrahedron Lett* **43**, 6525–8 (2002).
23 Palcic MM, Hindsgaul O, Glycosyltransferases in the synthesis of oligosaccharide analogs, *Trends Glycosci Glycotechnol* **8**, 37–49 (1996).
24 Elhalabi JM, Rice KG, Synthesis and applications for unnatural sugar nucleotides, *Curr Med Chem* **6**, 93–116 (1999).
25 Ohrlein R, Glycosyltransferase-catalyzed synthesis of non-natural oligosaccharides, *Top Curr Chem* **200**, 227–54 (1999).
26 Blanken WM, Van den E, Dirk H, Biosynthesis of terminal Gal$\alpha(1 \rightarrow 3)$Gal$\beta(1 \rightarrow 4)$GlcN Ac-R oligosaccharide sequences on glycoconjugates. Purification and acceptor specificity of a UDP-Gal: N-acetyllactosaminide α1,3-galactosyltransferase from calf thymus, *J Biol Chem* **260**, 12927–34 (1985).
27 Mazur A, Galactose Oxidase. In: *Enzymes in Carbohydrate Synthesis*, edited by Bednarski MD (Oxford University Press, New York, 1991), pp. 99–110.
28 Osawa T, Irimura T, Kawaguchi T, Bauhinia purpurea agglutinin, *Methods in Enzymology* **50**, 367–72 (1978).
29 Furka A, Sebestyen F, Asgedom M, Dibo G, General method for rapid synthesis of multicomponent peptide mixtures, *Int J Pept Protein Res* **37**, 487–93 (1991).
30 Ohlmeyer MHJ, Swanson RN, Dillard L, Reader JC, Asouline G, Kobayashi R, Wigler M, Still WC, Complex synthetic chemical

libraries indexed with molecular tags, *Proc Nat Acad Sci USA* **90**, 10922–6 (1993).

31 Itoh S, Taki M, Takayama S, Nagatomo S, Kitagawa T, Sakurada N, Arakawa R, Fukuzumi S, Oxidation of Benzyl Alcohol with CuII and ZnII Complexes of the Phenoxyl Radical as a Model of the Reaction of Galactose Oxidase, *Angew Chem Int Ed* **38**, 2774–6 (1999).

32 Sujino K, Malet C, Hindsgaul O, Palcic MM, Acceptor hydroxyl group mapping for calf thymus α1,3galactosyltransferase and enzymatic synthesis of α-D-Gal$p(1 \rightarrow 3)$-β-D-Gal$p(1 \rightarrow 4)\beta$ DGlc$p$NAc analogs, *Carb Res* **305**, 483–9 (1998).

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